Interaction between Human Immunodeficiency Virus Type 1 Reverse Transcriptase and Integrase Proteins

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Reverse transcriptase (RT) and integrase (IN) are two key catalytic enzymes encoded by all retroviruses. It has been shown that a specific interaction occurs between the human immunodeficiency virus type 1 (HIV-1) RT and IN proteins (X. Wu, H. Liu, H. Xiao, J. A. Conway, E. Hehl, G. V. Kalpana, V. R. Prasad, and J. C. Kappes, J. Virol. 73:2126–2135, 1999). We have now further examined this interaction to map the binding domains and to determine the effects of interaction on enzyme function. Using recombinant purified proteins, we have found that both a HIV-1 RT heterodimer (p66/p51) and its individual subunits, p51 and p66, are able to bind to HIV-1 IN. An oligomerization-defective mutant of IN, V260E, retained the ability to bind to RT, showing that IN oligomerization may not be required for interaction. Furthermore, we report that the C-terminal domain of IN, but not the N-terminal zinc-binding domain or the catalytic core domain, was able to bind to heterodimeric RT. Deletion analysis to map the IN-binding domain on RT revealed two separate IN-interacting domains: the fingers-palm domain and the carboxy-terminal half of the connection subdomain. The carboxy-terminal domain of IN alone retained its interaction with both the fingers-palm and the connection-RNase H fragments of RT, but not with the half connection-RNase H fragment. This interaction was not bridged by nucleic acids, as shown by micrococcal nuclease treatment of the proteins prior to the binding reaction. The influences of IN and RT on each other's activities were investigated by performing RT processivity and IN-mediated 3′ processing and joining reactions in the presence of both proteins. Our results suggest that, while IN had no influence on RT processivity, RT stimulated the IN-mediated strand transfer reaction in a dose-dependent manner up to 155-fold. Thus, a functional interaction between these two viral enzymes may occur during viral replication.

Reverse transcription is a key event in the replication cycle of human immunodeficiency virus type 1 (HIV-1) that is initiated after the virus enters the cell. Events subsequent to reverse transcription include nuclear transport of the preintegration complex (PIC), targeting PICs to the sites of integration, proviral integration, and finally the repair of the termini by mechanisms not yet fully understood. Reverse transcription is initiated in the cytoplasm, and the viral cDNA is synthesized in the cytoplasmic compartment. However, it is unclear if reverse transcription is completed prior to proviral integration, as proviral DNA containing discontinuities in plus-strand DNA has been shown to efficiently integrate in vitro. Indeed, reverse transcriptase (RT) has been detected in PICs and intracellular reverse-transcription complexes isolated from the nuclei of HIV-1-infected cells (5, 11). Thus, it is possible that RT is required for polymerization subsequent to nuclear transport, including the completion of plus-strand DNA synthesis and for repair of the single-strand gaps that remain after integration. In certain retrotransposons, exemplified by R2Bm, reverse transcription begins after endonucleolytic cleavage of the genomic DNA by integrase (IN). The RNA is copied using the 3′-OH terminus generated by cleavage of the genomic DNA (24), which would necessitate the retention of RT in the nuclear PICs.

Furthermore, in avian leukosis virus (14, 30) and in human T-lymphotropic leukemia virus type 1 (33), RT and IN are parts of a single polypeptide forming the α subunit of the heterodimeric αβ complex. In Rous sarcoma virus, where it has been well studied, the αβ complex retains both RT and IN activities. In addition, the recent demonstration that the αβ complex localizes to the nucleus suggests that the RT and IN proteins are likely present together in the nuclear PICs (37). In contrast, the RT and IN proteins of murine retroviruses and of HIV are fully separated by proteolytic cleavage during virion maturation (27). The observation that RT is present in the nuclear PICs (5) in addition to within reverse-transcription complexes (11) suggests that RT may be retained via protein-protein or protein-nucleic acid interactions with other viral components.

Previous studies led to the observation that the RT and IN proteins specifically interact with each other and that this interaction is not mediated by nucleic acid bridging (38). Here, we demonstrate that both monomeric and heterodimeric forms of RT can interact with IN, and we map the domains of interaction on both protein partners. Attempts to assess the effect of IN on RT function showed that RT function was unaffected by IN. In contrast, in the presence of RT, the IN-mediated joining reaction was stimulated significantly, while the 3′-end processing was unaffected. These results suggest functional interaction of the two proteins.

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MATERIALS AND METHODS

Bacteria and plasmids. The *E. coli* strain, M15::pDM1.1 (23) was used to express wild-type and mutant RTs and untagged HIV-1 RT and hexahistidine-tagged IN, and the strain BL21 {F⁻ *ompT gal* [*dcm*] [*lon*] *hsdSB* (r$_B^-$ m$_B^-$)}, an *E. coli* B strain, was used to express glutathione *S*-transferase (GST)-tagged wild-type and mutant HIV-1 IN proteins.

Heterodimeric and monomeric hexahistidine-tagged RT p66/p51, p66, and p51 proteins were expressed from plasmids pRT6H-PROT, p6HRT, and pRT6H51, respectively (21). The expression plasmid encoding IN from HIV-1$_{HxB2}$ was generated by ligating a 1.45-kb BamHI and SalI fragment from the yeast expression plasmid pSHIN (18) into pGEX-3XPL (a gift of D. Shore, University of Geneva, Geneva, Switzerland), yielding pGEX-IN. The HIV IN expression plasmids pT7IN and pT7ΔIN were provided by R. Swanstrom (University of North Carolina, Chapel Hill). GST was expressed from the vector pGEX-3XPL. The plasmid pHIV-PBS (2) was a gift of M. A. Wainberg (McGill University).

A maltose-binding protein (MBP)-IN fusion protein expression construct was prepared by inserting the IN fragment from pGEX-IN in frame with the MBP sequences in pMal c2.1 to yield the pMal c2.1-IN construct using BamHI and SalI sites.

Generation of RT and IN truncations and other mutations. Deletions in RT were generated by PCR amplification of sequences corresponding to each of the RT structural subdomains, with the borders of the domains as defined by Jacobo-Molina et al. (16). The primers used contained the restriction sites BamHI (upstream) and SalI (downstream) for cloning into the p6HRT plasmid. The domains that were subcloned included fingers and palm (FP; amino acids [aa] 1 to 242); fingers, palm (FPT; aa 1 to 322); thumb (T; aa 242 to 322), thumb-connection-RNase H (TCR; aa 242 to 560), connection and RNase H (Conn-R; aa 322 to 560), partial connection and RNase H (C*R; aa 387 to 560), and RNase H (R; aa 422 to 560), respectively. The PCR products were cloned into vectors, and the resulting plasmids were sequenced to ensure the absence of point mutations. The various GST-IN truncation mutants were obtained as follows. The IN deletions previously described, such as the core domain (48 to 208) or INΔ221–288 (18), were subcloned into the GST expression plasmid. The N-terminal and C-terminal domains of IN were PCR amplified from a proviral clone of HIV$_{Hxb2}$ using primers containing sequences complementary to the regions indicated immediately flanked by a BamHI site at the 5′ end and a stop codon immediately followed by SalI site at the 3′ end. The IN domain constructs used in this study were the zinc finger domain (aa 1 to 50), the core domain (aa 48 to 208), and the carboxy-terminal domain (201–288).

The GST-IN constructs containing the point mutations H12A, H16A, D116A, and F185A were all generated via site-directed mutagenesis using the QuikChange XL kit (Stratagene) as detailed in the manufacturer's instruction. The primer pairs used for generating each of these mutations (the bases corresponding to the mutant codon are shown in boldface) were as follows: H12A (5′-GGCCCAAGATGAA**GCT**GAGAAATATCACAGTAATTGGAG-3′ and 3′-CCGGGTTCTACTT**CGA**CTCTTTATAGTGTCATTAACCTC-5′); H16A (5′-GAACATGAGAAATAT**GCC**AGTAATTGGAGAGCAATGGC-3′ and 3′-CTTGTACTCTTTATA**CGG**TCATTAACCTCTCGTTACCG-5′), D116A (5′-GCCAGTAAAAACAATACAT**ACT**GCCAATGGCAGCAATTTC-3′ and 3′-CGGTCATTTTTGTTATGTA**TGA**CGGTTACCGTCGTTAAAG-5′), and F185A (5′-GCAGTATTCATCCACAAT**GCT**AAAAGAAAAAGGGGGGGAT T-3′ and 3′-CGTCATAAGTAGGTGTTA**CGA**TTTTCTTTTCCCCCCT AAC-5′). The presence of the desired mutations and the absence of the undesirable mutations were confirmed by sequencing.

The mutations W235A and W235E were created using PCR mutagenesis as follows. First, IN sequences were amplified in two parts: codons 1 to 235 and 236 to 288. The larger fragment was generated using the 5′-Fragment^{UP} and 5′-Fragment^{DOWN} PCR primers. The primer 5′-Fragment^{DOWN} contained one randomized position (equal proportions of all 4 nucleotides) in a nucleotide corresponding to codon 235 (the complement of the mutant codon shown in boldface below) to generate both an Ala codon (GCG) and a Glu codon (GAG) upon PCR mutagenesis. The downstream fragment contained no mutations. The restriction sites flanking the two IN fragments were BamHI and BsmBI (for the large fragment) and BsmBI and SalI (for the small fragment). The fragments were digested with appropriate restriction enzymes and cloned into pGEX or pMal c2.1 vectors in a three-fragment ligation. Twenty-four clones were sequenced to identify the desired mutants and to confirm the absence of undesired mutations. The oligonucleotide sequences, with the vector sequences in lowercase letters, were as follows: 5′-Fragment^{UP}, 5′-atcctaggatccccTTTTTAGATG GAATAGATAAGGCCC-3′; 5′-Fragment^{DOWN}, 3′-CTGTCGTCTTTAAGTG AAC**N**CTTTCCTGGTCGTTTCCTCTGC-5′; 3′-Fragment^{UP}, 5′-CGTCTCGC AAAGCTCCTCTGGAAAGGTGAAG-3′; and 3′-Fragment^{DOWN}, 3′-CACAC CGTTCATCTGTCCTACTCCTAATCcagctgcagtag-5′.

GST pull-down assay. Bacteria expressing all three forms of RT (p66, p51, and the p66/p51 heterodimer) were induced with 1 mM IPTG (isopropyl-β-D-thio-galactopyranoside) for 3 h, and the bacterial pellets were lysed with lysozyme in 50 mM HEPES (pH 7.2)–300 mM NaCl–0.1 mM phenylmethylsulfonyl fluoride (PMSF). Precleared lysates were used for interaction studies or stored at −70°C for later use. One-liter bacterial cultures expressing GST and GST-IN plasmids were pelleted and resuspended in 20 ml of buffer Y (0.2 M NaCl) consisting of 50 mM HEPES (pH 7.2), 200 mM NaCl, 1 mM EDTA, 0.5% IGEPAL CA-630 (a nonionic detergent), 0.3 μM aprotinin, 4 μM leupeptin, 2 μM pepstatin A, and 0.1 mM PMSF, and the lysates were prepared by six freeze-thaw cycles followed by lysozyme treatment. Glutathione-agarose beads (G beads) were prepared by resuspending them in Tris-buffered saline (25 mM Tris-Cl, pH 7.4, 136 mM NaCl, and 2 mM KCl) followed by two washes with water and one with buffer Y (containing 50 mM NaCl). The lysates (20 ml) were then bound to 0.4 ml of G-bead suspension. The protein-bound beads were washed extensively with buffer Y (0.2 M NaCl), and the bound proteins were quantitated by resolving them on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) alongside known quantities of bovine serum albumin (BSA), followed by Coomassie blue staining.

The binding reactions were carried out by mixing 50 μl of G beads and bound GST fusion proteins with 37 μl of RT lysate in 20 mM HEPES (pH 7.2)–120 mM NaCl–5 mM dithiothreitol (DTT)–4 mM MgCl$_2$–1 mM EDTA–0.5% IGEPAL CA-630–0.3 μM aprotinin–4 μM leupeptin–2 μM pepstatin A–0.1 mM PMSF at 4°C with gentle agitation. The beads were collected by centrifugation, washed six times with buffer Y to remove unbound proteins, resuspended in 2× SDS sample buffer, boiled, and subjected to SDS-PAGE analysis.

Micrococcal-nuclease treatment. GST, GST-IN, or GST-IN carboxy-terminal domain fusion protein bound to G beads was washed and quantitated on SDS-PAGE using known inputs of BSA, followed by Coomassie staining the gels. Approximately 1 μg equivalent of the bound proteins was resuspended in buffer Y and supplemented with calcium acetate to a final concentration of 1 mM, followed by the addition of 1.25 U of micrococcal nuclease (25 U/ml). The reactions were carried out at 37°C for 30 min and terminated by the addition of EGTA to a final concentration of 4 mM. The RT p66 and the deletions FP, Conn-R, and C*R were similarly treated with micrococcal nuclease. Approximately 200 μl of induced bacterial lysate containing the RT proteins was first supplemented with calcium acetate, followed by the addition of 5 U of micrococcal nuclease (25 U/ml), and reactions were carried out as described above. The GST pull-down assay was carried out using nuclease-treated binding partners as described above, and the bound proteins were separated on SDS-PAGE and subjected to immunoblot analysis using the appropriate anti-RT antibodies to detect the RT p66 or the deletion proteins. A duplicate gel of the binding reaction was run and Coomassie stained to confirm that equal inputs of the GST proteins were used in all reactions.

Hexahistidine pull-down assay. Nine milliliters of cleared lysates containing hexahistidine-tagged RT were incubated with 1 ml of Ni²⁺-nitrilotriacetic acid (NTA) agarose beads and prewashed three times with a wash buffer (50 mM Na-PO$_4$ [pH 8.0], 200 mM NaCl, 5 mM β-mercaptoethanol [βME], 20 mM imidazole, 0.3 μM aprotinin, 4 μM leupeptin, 2 μM pepstatin A, 0.1 mM PMSF). The binding reactions were set up by mixing 200 μl of a control IN-lacking (T7ΔIN) or IN-containing (T7IN) lysate with 100 μl of bound Ni²⁺ beads in a buffer containing 50 mM Na-PO$_4$ (pH 7.0), 200 mM NaCl, 5 mM βME, 5 mM imidazole, 0.3 μM aprotinin, 4 μM leupeptin, 2 μM pepstatin A, 0.1 mM PMSF, and 10 mg of BSA/ml. The reaction mixtures were incubated at 4°C for 1 h with gentle agitation, washed three times with wash buffer, mixed with 2× SDS sample buffer, and resolved by SDS-PAGE.

Immunoblot analysis. Proteins resolved by SDS-PAGE were transferred to nitrocellulose and probed with one of the anti-RT (α-RT) monoclonal antibodies 8C4D7, 5B2B2, and 7E5E6 (31), which recognize epitopes common to both p66 and p51 polypeptides, or the polyclonal α-HIV-1 HXB2 IN antiserum (directed against N-terminal residues 23 to 34; provided by D. Grandgenett, St. Louis University Health Sciences Center, St. Louis, Mo.) (12).

Purification of IN protein. Purified HIV-1 IN for the processing and joining reactions was obtained by inducing 1 liter of p6H-IN in M15::pDM1.1. After the cultures were pelleted and lysed with 20 ml of lysis buffer (50 mM NaPO$_4$, pH 7.4, 0.1 mM EDTA, 5% glycerol, 10 mM βME, 1 mM PMSF, 20 mg of lysozyme), the lysates were supplemented with 1.16 g of NaCl, sonicated three times, and passed through a 21.5-gauge needle. The lysates were then mixed with an equal volume of lysis buffer to reduce the NaCl concentration to 0.5 M, followed by centrifugation at 35,000 rpm (Optima LE 80K; Beckman Coulter) for 30 min and binding of the supernatants to Ni²⁺-NTA agarose. The columns were washed

with 50 mM NaPO$_4$ (pH 6.3), 0.1 mM EDTA, 5% glycerol, 10 mM βME, 1 mM PMSF, 0.5 M NaCl, and 5 mM imidazole and eluted with 50 mM NaPO$_4$, pH 7.4, 0.1 mM EDTA, 5% glycerol, 10 mM βME, 1 mM PMSF, and 0.5 M NaCl in the presence of an imidazole gradient. The protein was then dialyzed against 20 mM HEPES, pH 7.5–1 mM DTT–1 mM EDTA–0.5 M NaCl–20% glycerol.

3′ processing-strand transfer reactions of IN. The model DNA substrates for IN-mediated 3′-end processing and joining reactions were as described previously (28). For 3′-end processing, a pair of DNA oligonucleotides, U5.3 (5′-GGATCCGGAAAATCTCTAGCAGT-3′) and U5.4, (5′-ACTGCTAGAGATTTTCCGGATCC-3′) were used. For joining reactions, U5.4 was used with the oligonucleotide U5.5 (5′-GGATCCGGAAAATCTCTAGCA-3′), which mimics a preclipped version of U5.3. The oligonucleotides U5.3 and U5.5 were 5′ end labeled and separately annealed to U5.4. For each processing and joining reaction, 0.1 pmol of U5.3/U5.4 or U5.5/U5.4 was used.

The 3′-end processing-joining reactions were performed in either IN buffer (25 mM HEPES [pH 7.2], 50 mM NaCl, 7.5 mM MnCl$_2$, 10 mM βME, 0.1 mg of BSA/ml, 3% glycerol) or RT-IN buffer, which was optimized for RT-IN interaction (20 mM HEPES [pH 7.2], 120 mM NaCl, 4 mM MnCl$_2$, 5 mM DTT, 0.1 mg of BSA/ml, 3% glycerol). The 3′-end processing reactions were set up by mixing 4.68 pmol of IN with various amounts of RT ranging from a 32:1 to a 1:2 molar ratio of IN monomer to RT heterodimer. The joining reactions were set up by mixing a constant input of the IN protein (4.68 pmol) with various amounts of RT ranging from an 8:1 to a 1:1 molar ratio of IN monomer to RT heterodimer. After the addition of both IN and RT, the reaction mixtures were preincubated at room temperature for 5 min. The IN reactions were initiated by the addition of 1 μl of processing substrate (0.1 pmol of radiolabeled U5.3/U5.4) or joining substrates [0.1 pmol of U5.4/U5.5 and 0.2 μg of pBluescript SK(+)]. The reactions were carried out for 1 h at 37°C.

Measurements of RT processivity. The effect of IN on the processive polymerization of RT was assessed by using an RNA template generated from pHIVPBS. The in vitro transcription of pHIVPBS was carried out using an Ambion Megascript kit, which produced an RNA that corresponds to nucleotides 473 to 1444 of HIV$_{HXB2}$. A 28-mer DNA primer (corresponding to positions 662 to 635 of HIV$_{HXB2}$), 5′-CGCTTTCAGGTCCCTGTCCGGGCGCC AC-3′, was ^{32}P end labeled and annealed to the RNA template. The reactions, in a total volume of 25 μl, were carried out as follows. A constant input of purified HIV-1 RT (5.5 pmol) and various amounts of IN (0.17 to 22 pmol) were incubated with the template-primer (1 μM) prepared as described above for 5 min at 37°C in 1× RT-IN reaction buffer (20 mM Tris-Cl, pH 7.0, 120 mM KCl, and 5 mM DTT). The RT reactions were started by the addition of 5 μl of cocktail containing poly(rA)·oligo(dT) as an enzyme trap (heparin) and deoxynucleoside triphosphates (dNTPs) to initiate the reaction. After incubation for 15 min at 37°C, the reactions were terminated and resolved on 5% PAGE gels under denaturing conditions.

RESULTS

IN can bind to the heterodimeric p66/p51 and monomeric p66 and p51 RTs. It has been reported that the GST-IN fusion protein is able to interact directly with heterodimeric hexahistidine-tagged p66/p51 RT present in bacterial lysates (38). In that report, the authors also showed that the interaction is not bridged by nucleic acids, as demonstrated by pretreatment of both interacting partners with micrococcal nuclease prior to mixing them. In the present study, we wanted to determine if p66, which is known to exist in a homodimeric form, and p51 monomers also bind to HIV-1 IN. We expressed hexahistidine-tagged p51, p66, and p66/p51 RTs and untagged IN, all derived from HIV-1$_{HXB2}$ in bacteria. Each preparation of RT was bound to Ni^{2+}-agarose beads, washed, and incubated with bacterial lysates containing IN. After the beads were washed to eliminate unbound proteins, the bound proteins were separated by SDS-PAGE, followed by immunoblot analysis with α-IN antibodies. An identical sample of bound proteins separated on SDS-PAGE was transferred to nitrocellulose and probed with α-HIV-1 RT antibodies to ensure that the amounts of bound RT proteins in all reaction mixtures were comparable. The results indicated that each of the three forms
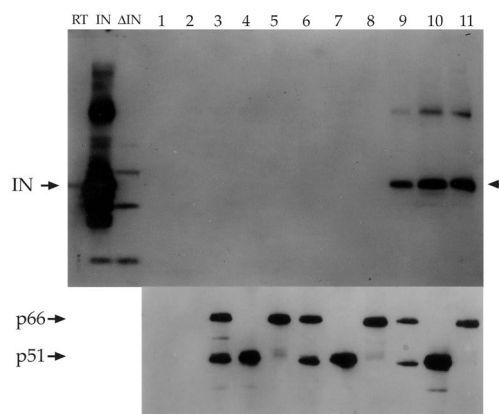

FIG. 1. Native IN interacts with all three forms of HIV-1 RT. Hexahistidine-tagged RT heterodimer (p66/p51), p51, and p66 bound to Ni^{2+}-NTA agarose beads were incubated with crude bacterial lysates containing native IN. The beads were washed, and the bound proteins were resolved by SDS–15% PAGE. (Top) Immunoblot probed with polyclonal α-IN antibodies directed to N-terminal residues 23 to 34. Lanes RT, IN, and ΔIN contain unreacted lysates from bacteria (RT), the IN expression plasmid (pT7IN), and the expression plasmid without the IN sequences (pT7ΔIN). Lanes 1 and 2, empty Ni^{2+}-NTA beads incubated with pT7ΔIN lysate or pT7IN lysate; lanes 3 to 5, Ni^{2+}-NTA beads bound to p66/p51, p51, or p66; lanes 6 to 8, Ni^{2+}-NTA beads bound to p66/p51, p51, or p66 incubated with pT7ΔIN lysate; lanes 9 to 11, Ni^{2+}-NTA beads bound to p66/p51, p51, or p66 incubated with pT7IN lysate. (Bottom) Parallel protein transfer blot probed with the α-RT antibody 8C4D7 to ensure the presence of similar input RT bait protein. Lanes 1 to 11 are identical to those in panel A.

of RT was able to bind to IN protein from the bacterial lysates (Fig. 1, top). The presence of comparable inputs of RT proteins in the reaction mixtures was verified by immunoblot analysis of a duplicate gel with the α-RT monoclonal antibody 8C4D7 (Fig. 1, bottom).

Multimerization of IN is not required for binding to RT. The formation of multimers of IN is known to be essential for IN function (9, 10, 12, 18, 35). Therefore, it was of interest to determine if the ability of IN to multimerize was necessary for association with RT. A multimerization-defective mutant of IN with a V260E substitution, isolated via a two-hybrid screen, was previously reported (20). This mutant exhibited a reduced ability to interact with wild-type IN both in the two-hybrid system and in vitro as a GST fusion protein but retained the ability to interact with INI1/hSNF5, indicating that it is specifically defective for homomeric interactions but not for heteromeric interactions. Furthermore, viruses containing a V260E mutation were defective for replication, suggesting that IN multimerization is important for IN function. We tested the ability of GST-IN or GST-INV260E bound to G beads to interact with p66 in bacterial lysates using the same conditions described above. The results indicated that the V260E mutant of IN was able to bind to RT as effectively as wild-type IN (Fig. 2A). Thus, disruption of IN multimerization does not appear to affect the ability of IN to interact with RT, indicating that IN multimerization may not be required for this interaction.

Determination of IN-binding domain on RT. To determine the domain of RT that is necessary and sufficient to bind to IN, we expressed a panel of N- and C-terminal deletions of RT
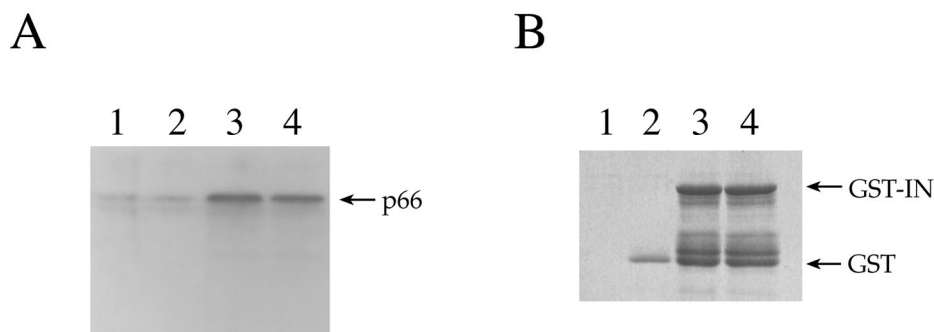
FIG. 2. Multimerization of IN is not critical to RT-IN interaction. (A) Lanes 1 to 3, empty, GST-bound, and GST-IN-bound G beads incubated with bacterial lysates containing RT p66; lane 4, GST-IN/V260E-bound G beads incubated with p66 RT lysate. The immunoblot was probed with the α-RT antibody 5B2B2. (B) Parallel SDS-PAGE gel from the experiment shown in panel A Coomassie stained to ensure the presence of equivalent amounts of the proteins.

corresponding to FP, FPT, T, TCR, Conn-R, C*R, and R (Fig. 3B). These fragments were each expressed with an N-terminal hexahistidine tag in bacteria and tested for interaction in vitro using GST-IN bound to glutathione agarose beads. The bound proteins were analyzed both by probing an immunoblot with anti-HIV-1 RT antibodies to detect the truncated RT proteins that retain binding and by Coomassie staining to assess the amount of GST-IN protein in the reactions. The results demonstrated that two nonoverlapping fragments bordering the polymerase domain were each able to bind to IN, indicating that there are two IN-binding sites on RT. One of the binding sites resides in the FP domain (Fig. 3A, lanes 14 to 17), and another resides in the C-terminal domain of connection between aa 387 and 422 (Fig. 3A, lanes 3 to 6, and B, bottom).

Determination of RT-binding domain on IN. To map the domain of IN necessary and sufficient to bind to RT, we expressed GST fusion proteins of each of the three structural domains of IN: the zinc-binding, the catalytic core, and the carboxy-terminal domains. The lysates containing each of these fusion proteins were then bound to G beads and subjected to interaction with the heterodimeric form of RT. The proteins bound to GST-IN fragments were resolved by SDS-PAGE and analyzed by immunoblotting them with monoclonal α-RT 5B2B2 antibodies (Fig. 4). The results indicate that while neither the zinc-binding domain nor the central core was capable of interacting with RT, the C-terminal domain of IN could bring down RT as efficiently as the full-length IN. To confirm the results showing that the C-terminal domain is the RT-binding domain, we tested the interaction of a GST fusion of a mutant of IN with a deletion of the carboxy-terminal domain (ΔIN, retaining amino acid residues 1 to 220) with RT. The results indicated that ΔIN was unable to bind to RT, confirming that the RT-binding site resides in the C-terminal domain of IN (Fig. 4, lane 10). Two other deletions lacking 80 and 82 residues from the carboxy terminus were also defective for RT interaction (data not shown).

Interaction between the smallest interacting fragments of RT and IN. The results described above showed that the carboxy-terminal domain of IN can interact with full-length RT and that the FP and Conn-R fragments can both interact with full-length IN fused to GST. It was unclear whether the carboxy-terminal domain of IN alone can bind to RT fragments encoding the FP or the Conn-R domain. Therefore, we tested

the abilities of the GST-IN and GST–C-terminal IN proteins to bind p66, Conn-R, or C*R expressed in bacteria. Our results, presented in Fig. 5, show that both GST-IN and the GST–C-terminal IN proteins can pull down p66, as well as its deletion proteins containing FP and Conn-R. Interestingly, however, C*R could be brought down by GST-IN but not by GST–C-terminal IN, suggesting that other regions of IN may be required for interaction with this region of RT either directly or indirectly. Equivalent amounts of the GST-IN and GST–C-terminal IN proteins were used in these experiments, as determined by Coomassie staining of a parallel SDS-PAGE gel (a 4 to 20% gradient acrylamide gel) of the same proteins used in the binding reaction (data not shown).

Interaction of minimal domains of RT and IN is not bridged by nucleic acids. Since all the structural domains involved (from both the interacting proteins), FP and Conn-R of RT and the C-terminal domain of IN, contain nucleic acid-binding domains, the question is whether the interaction observed is mediated by nucleic acid bridges. To address this issue, we performed a parallel reaction along with the above-mentioned experiment in which each interacting partner was pretreated with micrococcal nuclease prior to being adding to the in vitro binding reaction. Our results (compare Fig. 5A and B or E and F [+micrococcal nuclease] with C and D or G and H [−micrococcal nuclease]) indicate that the interaction of the minimal domains was unaffected by micrococcal-nuclease pretreatment, thus confirming the absence of nucleic acid bridging between the interacting partners.

Substitutions at the W235 residue do not prevent RT-IN interaction. In an earlier report, Ishikawa et al. stated that monoclonal antibodies directed to the C-terminal domain of IN prevented interaction between the HIV-1 RT and IN proteins and that the RT-IN interaction was disrupted by W235A and W235E substitutions in the IN protein (15). Therefore, we generated GST fusions of these mutant proteins and tested their abilities to pull down RT as described above. Surprisingly, both of the mutants were able to associate with RT as efficiently as the wild-type IN protein in the GST pull-down assays (data not shown). In evaluating the basis for this discrepancy, we noted that Ishikawa et al. used an MBP fusion of IN protein for their pull-down reactions. It is formally possible that our inability to reproduce their results was due to the use of a fusion partner here (GST) different from that used by Ishikawa
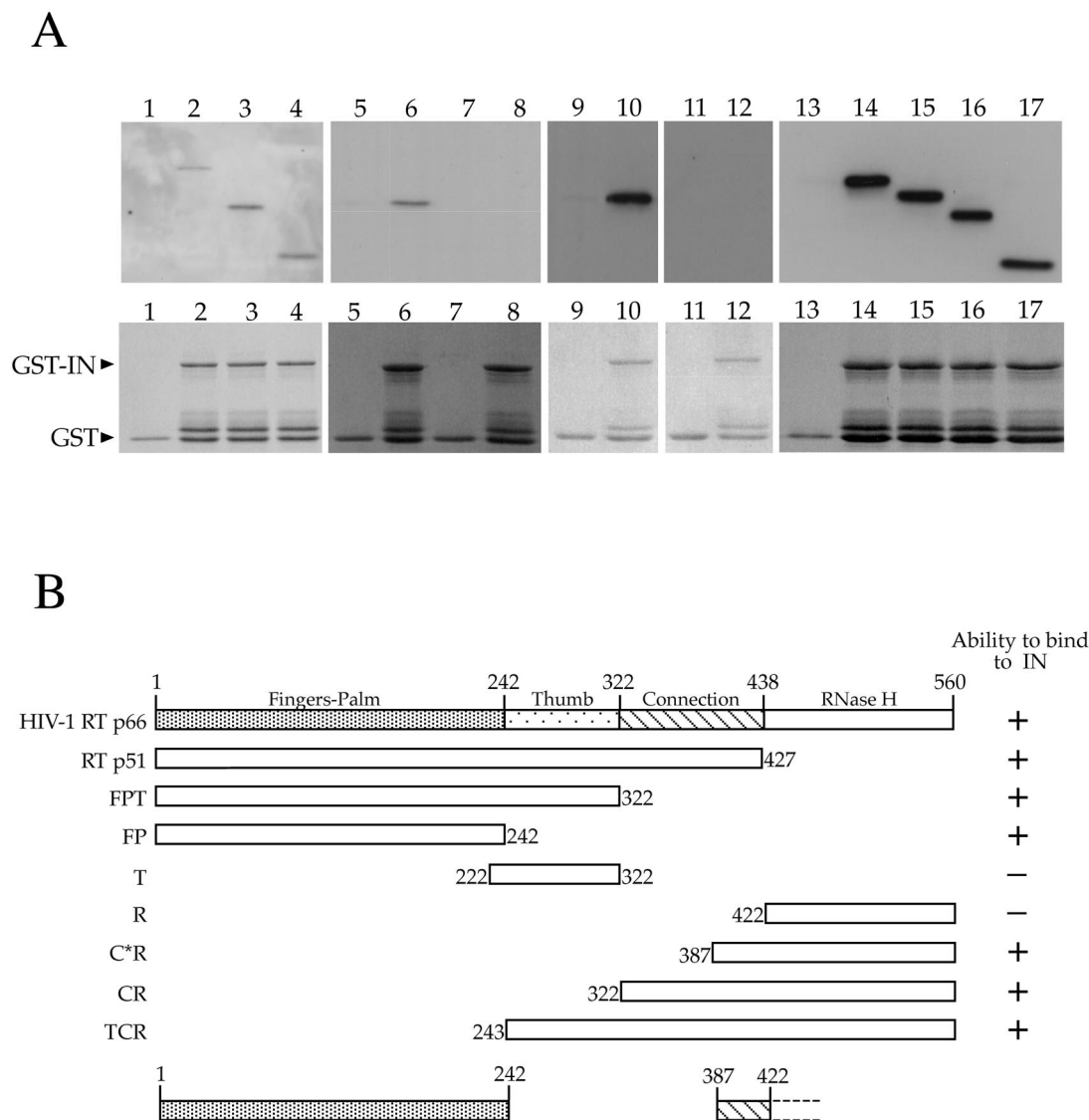
A

B

FIG. 3. Mapping the IN-binding domain on HIV-1 RT. In experiments similar to those shown in Fig. 2, G beads bound to GST or GST-IN were incubated with bacterial lysates containing various truncation mutants of RT and washed, and the bound proteins were resolved on duplicate SDS-PAGE gels. The proteins on one gel were transferred to nitrocellulose and subjected to immunostaining with α-RT antibodies, and the second gel was stained with Coomassie blue to ensure equal input of the bait proteins. (A) Pull-down experiments to map the IN-binding domains on RT p66. Lanes 1, 5, 7, 9, 11, and 13 contained control proteins from a control incubation of GST bound to G beads, and lanes 2 to 4, 6, 8, 10, 12, and 14 to 17 contained proteins bound to GST-IN bound to G beads. RT mutants were added to the lanes as follows: lane 2, p66; lane 3, TCR; lane 4, Conn-R; lanes 5 and 6, C*R; lanes 7 and 8, R; lanes 9 and 10, p66; lanes 11 and 12, T; lanes 13 and 14, p66; lane 15, p51; lane 16, FPT; and lane 17, FP. The corresponding lanes on the bottom show stained protein indicating the input bait protein levels. The differences in p66 intensities in lanes 2 and 14 are due to different antibodies used for the Western blots. (B) A schematic summarizing results obtained in the experiment shown in panel A. The horizontal bar at the top represents full-length RT p66, with various subdomains and their boundaries indicated by amino acid residue numbers. The RT truncations used in the pull-down experiment, their boundaries, and their abilities to bind IN are indicated. Below, the proposed domains of IN interaction are indicated. +, able to bind; −, not able to bind.

et al. (MBP). Therefore, we created MBP fusions of W235A and W235E IN mutants and used them in a pull-down assay to bring down heterodimeric RT from crude bacterial lysates, followed by analysis of the bound proteins. Our results show that MBP fusions of both of the mutant proteins bound to the RT heterodimer, indicating that these mutations do not disrupt RT-IN interaction (Fig. 6).

Mutations in conserved residues of IN do not affect RT interaction. Previous studies showed that mutations in the conserved residues of IN, such as H12A, H16A, F185A, and Δ22, affect HIV replication at the level of reverse transcription. Therefore, it was of interest to determine whether these mutations disrupted RT-IN interaction. We performed GST-IN pull-down experiments using each of these mutant IN proteins. As illustrated in Fig. 7, our results show that none of the mutations we tested has any effect on the ability of GST-IN to pull down RT from crude lysates. These results suggest that the defect in reverse transcription exhibited by mutant viruses
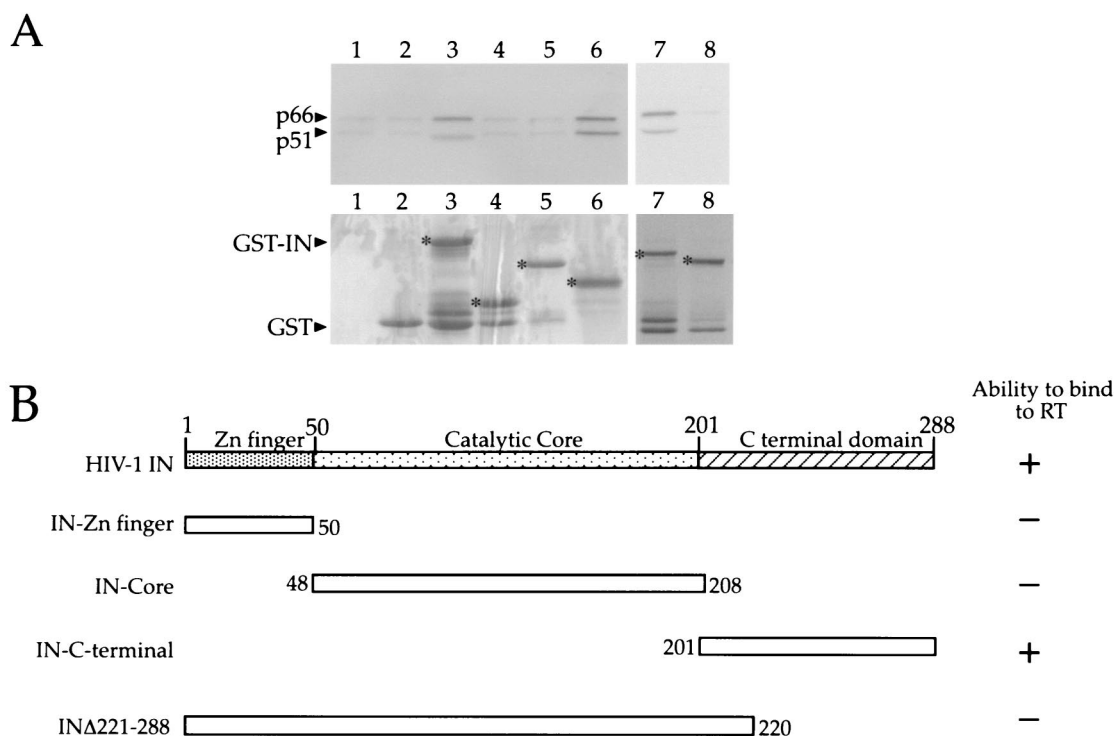
FIG. 4. Mapping the RT-binding domain on IN. (A) In an experimental setup for pull-down experiments similar to that in Fig. 3, GST-IN and GST fusions of various truncations of IN were incubated with heterodimeric RT, followed by washing and resolving the bound proteins on SDS-PAGE. As before, one gel was transferred to nitrocellulose and probed with monoclonal α-RT antibody 5B2B2 (top), and a duplicate gel with the same proteins was stained with Coomassie blue (bottom). Lane 1, empty G beads; lane 2: GST-bound G beads; lanes 3 and 7, GST-IN-bound G beads; lanes 4, 5, 6, and 8, G beads bound to GST-IN Zn finger domain (amino acid residues 1 to 50), GST-IN catalytic core domain (residues 48 to 208), GST-IN C-terminal domain (residues 201 to 288), and GST-IN C-terminal deletion (residues 1 to 220), respectively. (B) Schematic showing a summary of RT-binding abilities of full-length IN and the various truncation mutants of IN tested. The amino acid residues of each mutant are shown at the left of the horizontal bar representing each deletion. +, able to bind; −, not able to bind.

bearing these mutations is due to reasons other than the lack of RT-IN interaction. Furthermore, these results are consistent with our data showing that RT binds to the C-terminal domain but not the Zn-binding or core region of IN.

The effects of IN and RT on each other's catalytic functions. Mutations in HIV-1 IN have been shown to block viral replication at the level of reverse transcription (38). This influence of IN on reverse transcription may be mediated by RT-IN interaction. To evaluate the functional significance of the interaction, we first tested the effect of IN on RT function. In standard RT reactions to measure the level of incorporation of dNTP into DNA by RT, the addition of the IN protein at various levels had no effect (data not shown). In order to detect qualitative differences, we measured processive synthesis by RT in the presence or absence of the IN protein in the reactions. Using a constant input of RT, the amount of IN was varied to get ratios of RT heterodimer to IN monomer ranging from 1:0.25 to 1:32. After the reaction, the products were separated on a denaturing gel and exposed to autoradiography. Our results show that IN does not influence RT activity either on homopolymeric (data not shown) or on heteropolymeric RNA templates and does not influence its processivity (Fig. 8).

To determine the effect of RT on IN activity, we first carried out 3′ processing reactions in the presence of RT. As before, to ensure that the reaction buffer was optimally suited for RT-IN interaction, we compared 3′ processing in both the standard IN

buffer and a buffer optimized for RT-IN interaction. Since the 3′ processing activity of IN was undetectable in RT-IN buffer (Fig. 9A, lane 8), we used standard IN buffer conditions, under which significant levels of processing activity could be detected (Fig. 9A, lane 4). Under these reaction conditions, we found that the addition of RT (either 1:1 or 1:4 ratios of IN to RT) inhibited 3′ processing by IN. In order to determine the minimal amount of RT sufficient to inhibit 3′ processing, we performed a dose-dependence assay of this inhibition by varying the proportion of RT to a constant input of 4.68 pmol of IN. The molar ratios of IN monomer to RT heterodimer were 1:0.03125, 1:0625, 1:0.125, 1:0.25, 1:0.5, 1:1, and 1:2. The presence of RT appeared to inhibit the 3′ processing activity (Fig. 9B) in a dose-dependent manner. The lowest molar ratio of IN monomer to RT heterodimer sufficient to detect inhibition appeared to be 1:0.0625.

We then examined whether RT influenced the strand transfer or joining activity of IN. The RT concentration was varied from a 1:0.125 to a 1:1 molar ratio of IN monomer to RT heterodimer, maintaining IN at a constant concentration (Fig. 10A, lanes 4 to 7). The reactions were carried out using a supercoiled plasmid DNA as the recipient and oligonucleotide substrates labeled at the 5′ end of the joining strand. Insertion of oligonucleotide substrate into the target would result in a tailed plasmid DNA (lariat) that is radioactive and would be apparent on an autoradiogram. While the insertion of a single
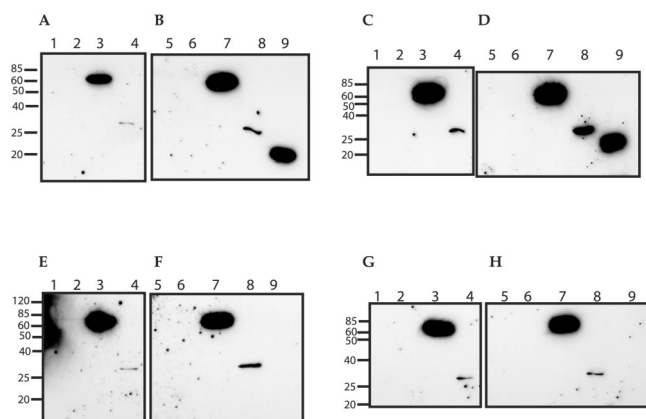
FIG. 5. Interaction between the binding domains occurs without nucleic acid bridging. G beads, GST, or GST-IN prepared with or without micrococcal-nuclease treatment were incubated with p66, FP, Conn-R, or C*R similarly prepared with or without micrococcal-nuclease treatment. (A) Pull-down reactions using micrococcal-nuclease-treated interaction partners. Plain G beads (lane 1) or G beads bound to 1 μg of GST (lane 2) or GST-IN (lane 3) were incubated with p66 protein. G beads bound to GST-IN alone were incubated with the FP domain (lane 4). Subsequent to the pull-down and SDS-PAGE (15% acrylamide) analysis of the bound proteins, an immunoblot was prepared and probed with the monoclonal antibody 8C4D7, which recognizes the epitope located between RT residues 193 and 284, which overlaps with the FP domains. (B) Pull-down reactions using micrococcal-nuclease-treated interaction partners. Similar to panel A, plain G beads (lane 5) or G beads bound to GST (lane 6) or GST-IN (lane 7) were incubated with p66. The G beads bound to GST-IN were also separately incubated with Conn-R (lane 8) or C*R (lane 9). Although both panels A and B are from the same SDS-PAGE gel, the immunoblot corresponding to panel B was probed with a different monoclonal antibody directed to the R domain (residues 440 to 560 of RT p66) to facilitate detection of the C-terminal fragments of RT. (C and D) The lanes are identical to those in panels A and B, except that none of the proteins were treated with micrococcal nuclease. (E and F) The lanes are similar to those in panels A and B, except that GST–C-terminal IN was used instead of GST-IN. All proteins were also treated with micrococcal nuclease. (G and H) The lanes are similar to those in panels E and F in that GST–C-terminal IN was used for pull downs, but no proteins were treated with micrococcal nuclease. The numbers on the left of the panels are molecular weight markers (prestained Invitrogen Benchmark).

oligonucleotide substrate (nonconcerted integration) into the target DNA results in the lariat structures, the concerted integration of two oligonucleotide substrates into the same target DNA results in a linearized plasmid DNA molecule. We found that the addition of low inputs of RT (as low as fourfold-lower molar proportion compared to IN) significantly stimulated the joining activity of IN (Fig. 10A and B, compare lanes 3 and 4). The above-mentioned reactions were carried out using the optimized buffer conditions for IN reactions. To determine if reaction buffers conducive to RT-IN binding would enhance the stimulation observed in IN buffer, we carried out the IN strand transfer reactions in buffers that facilitated maximal interaction of the two proteins. Although IN exhibited barely detectable activity in the absence of RT under these conditions, the presence of RT resulted in a dramatic increase (155-fold) in its activity (Fig. 10B, lanes 3 and 4). However, we did not see any linear products, suggesting that RT did not stimulate concerted integration. Based on these results, RT ap-

pears to stimulate IN joining activity while inhibiting 3′ processing activity.

DISCUSSION

Previous reports have demonstrated protein-protein interactions of retroviral RT and IN proteins, which are derived from a single polypeptide in the virus (14, 15, 32, 38) (39). Here, we describe the domains necessary for HIV-1 RT and IN interactions and the influence of each on the other's function in vitro. Our data demonstrate that in addition to heterodimeric RT, the p66 homodimers and p51 monomers can also interact with IN. This implies that IN recognizes a feature that is common to all three preparations of HIV-1 RT. The p66 preparations of RT are known to be predominantly in a homodimeric form and display up to 70% activity of heterodimeric RT (4, 23). Structurally, it is thought that one of the p66 molecules in the homodimer assumes the shape of p51 (36). However, the p51 molecule is known to exist mostly as a monomer in the absence of p66 and homodimerizes only under certain unusual conditions, including a high protein concentration (8). The conformation of monomeric p51 is unknown, but
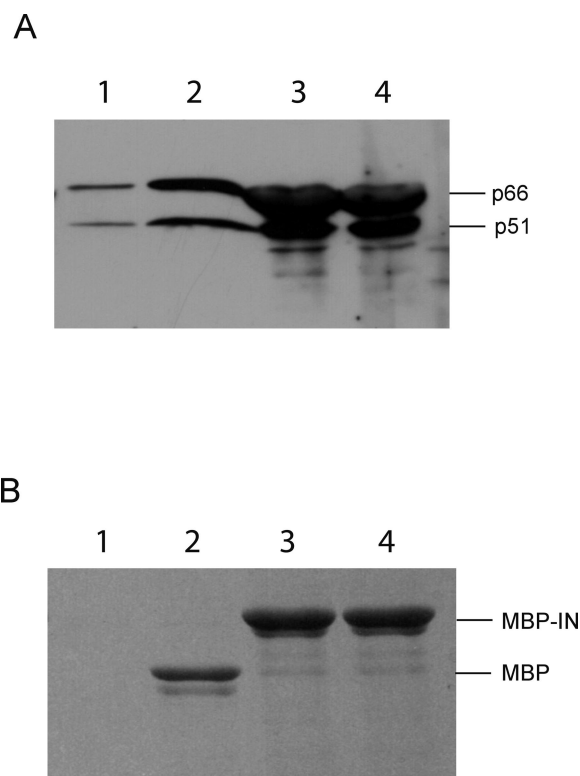

FIG. 6. W235 substitutions do not disrupt RT-IN interaction. MBP-IN fusion proteins containing W235A or W235E mutations were concentrated from induced lysates by using amylose resin beads. Amylose resins alone (lanes 1) and amylose bound to MBP (lanes 2), MBP-IN^{W235A} (lanes 3), or MBP-IN^{W235E} (lanes 4) protein were incubated with lysates containing HIV-1 RT heterodimer followed by washing the resin and analysis of bound proteins on SDS-PAGE. (A) Immunoblot analysis using α-RT antibodies. The positions of 66- and 51-kDa subunits of RT are indicated. (B) Gel identical to that in panel A stained with Coomassie brilliant blue. The migration positions of the MBP-IN fusion and MBP are indicated.
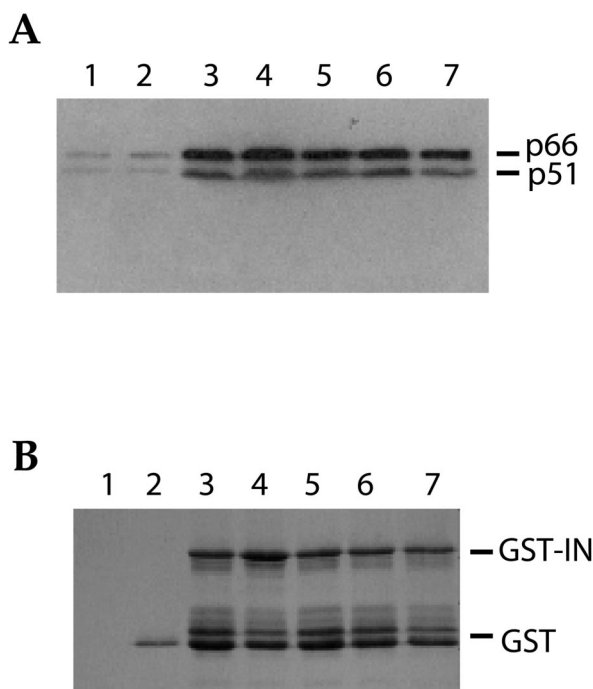
FIG. 7. Effects of mutations at conserved IN residues on RT-IN interaction. GST pull-down experiments were carried out as before by incubating wild-type GST-IN or the substitution mutants H12A, H16A, D116A, and F185A with lysates containing heterodimeric RT. (A) Bound proteins were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with the α-RT antibody 5B2B2. Lanes 1 to 3, empty, GST-bound, and GST-IN-bound G beads; lanes 4 to 7, GST-IN–H12A, GST-IN–H16A, GST-IN–D116A, and GST-IN–F185A, respectively. The positions of p66 and p51 polypeptides are indicated. (B) A parallel SDS-PAGE gel was Coomassie blue stained to ensure the presence of equivalent inputs of proteins. The positions of GST and GST-IN are indicated.

subdomain nor the R domains, when expressed alone, were able to bind IN. Furthermore, the minimal RT domains were able to bind to the GST-IN protein even when pretreated with micrococcal nuclease, demonstrating the absence of nucleic acid bridging in the interaction. More interestingly, when GST–C-terminal IN was used instead of the GST-IN protein in pull-down reactions, while both the FP and Conn-R fragments of RT retained interaction, C*R, which binds to GST-IN, showed no interaction with the GST–C-terminal IN protein. It is possible that regions outside the carboxy-terminal domain of IN used here (residues 201 to 288) may be required for interaction with the C*R domain. Whether the role of any such residues is in the direct interaction with RT or indirectly in modulating the overall conformation of the C-terminal domain of IN used here is unclear.

if one assumes that the monomeric p51 has a structure similar to that of the p51 present in a heterodimer, p51 would be the common element in all three preparations, and thus it is possibly the subunit with which IN interacts. Our data, however, do not rule out the involvement of the catalytic p66 subunit in the interaction.

Our results indicate that IN binds to two discontinuous regions on RT, the FP region (amino acid residues 1 to 242) and the carboxy-terminal half of the connection subdomain (amino acid residues 387 to 422). We have attempted to further delineate the binding site within the FP region by expressing smaller segments within the region. In the primary sequence of the RT protein, the FP region is not continuous but rather intermixed and arranged as Fingers1-Palm1-Fingers2-Palm2 (F1-P1-F2-P2) sequences. Unfortunately, all of the smaller segments of FP subdomains (F1-P1, F2-P2, etc.), when expressed alone, led to unstable proteins that could barely be detected or that were expressed at levels that were too low to facilitate binding studies.

Both of the two binding regions mentioned above (FP and half connection), when independently expressed as the entire FP or as half connection-RNase H domains, were able to bind to IN. This result was confirmed by testing nested deletions from both the amino and carboxy termini of RT. Neither the T
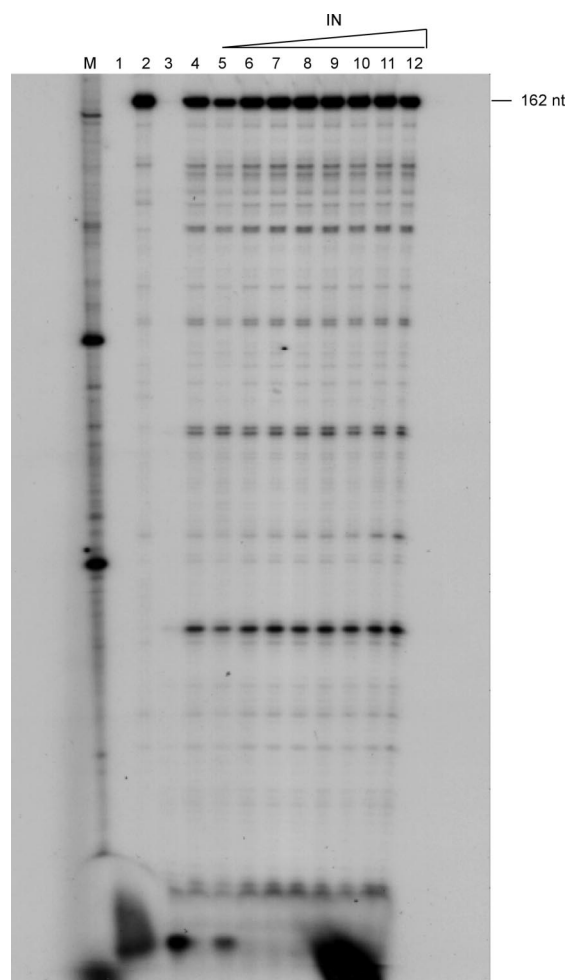

FIG. 8. Effect of IN on the processivity of HIV-1 RT. Using a primer that binds to the primer binding site, processivity reactions were done in the presence or absence of IN. Lane 1, primer alone; lane 2, untrapped reaction in the absence of IN; lane 3, pretrapped reaction where RT was mixed with poly(rA)·oligo(dT) prior to the addition of template-primer to ensure the effectiveness of the trap; lane 4, reaction in which dNTPs and trap were added simultaneously; lanes 5 to 12, reactions in which dNTPs and trap were added simultaneously in the presence of increasing amounts of IN ranging from a 1:0.03125 to a 1:4 molar ratio of RT to IN (with a constant input of RT). nt, nucleotides.
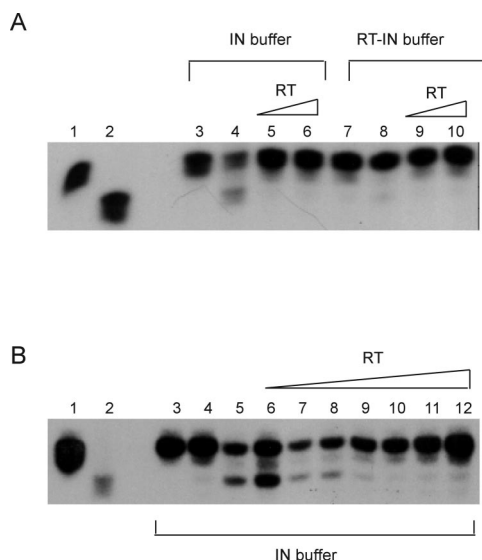
FIG. 9. Effect of RT on 3′ processing by IN protein. (A) Using radiolabeled U5.3/U5.4 substrate, a comparison was made between normal IN buffer and RT-IN buffer. Lane 1, unclipped U5.3/U5.4; lane 2, U5.5/U5.4, the preclipped version of U5.3/U5.4; lane 3, 3′ processing reaction using IN buffer in the absence of IN; lane 4, processing reaction using IN buffer in the presence of IN; lanes 5 and 6, processing reactions using IN buffer in the presence of IN and RT in a 1:1 to 1:4 molar ratio of IN monomer to RT, respectively; lanes 7 to 10, exactly as for lanes 3 to 6 except using RT-IN buffer. The reactions in lanes 4 to 6 and 8 to 10 were performed with a constant input of IN. (B) All of the 3′ processing reactions were done using normal IN buffer. Lanes 1 and 2, same as for panel A; lane 3, 3′ processing reaction in the absence of IN; lane 4, 3′ processing reaction in the absence of IN and the presence of 9.36 pmol of RT; lane 5, 3′ processing reaction in the presence of 4.68 pmol of IN monomer; lanes 6 to 12, 3′ processing reactions in the presence of 4.68 pmol of IN monomer and the following concentrations of RT: 1:0.03125, 1:0.0625, 1:0.125, 1:0.25, 1:0.5, 1:1, and 1:2 (IN monomer to RT heterodimer), respectively.

The inability of some of the RT fragments to bind IN may result from improper folding. We believe this to be unlikely, as several of the RT fragments utilized in our studies (FP, FPT, T, TCR, Conn-R, C*R, and R) have led to the reconstitution of a functional enzyme when used in mixing experiments (29). For example, when the Conn-R domain alone or the TC fragment was added to the enzymatically inactive C*R fragment, it resulted in reactivation of R. Furthermore, Unge et al. crystallized the FP domain from amino acid residues 1 to 216 and found it to be very close to the structure of the FP domain observed in p66 (34). Similarly, the crystal structure of a fragment of HIV-1 RT corresponding to the R domain is largely comparable to the structure of this domain when the entire heterodimer was crystallized (7, 16). Therefore, we believe that the fragments of RT used in our mapping experiments are likely to have been properly folded.

It is interesting that the IN-binding site maps to more than one subdomain of RT. If, as described above, one assumes that the p51 subunit mediates this interaction as discussed above, the structure of the p51 subunit shows the FP and Conn-R subdomains to be physically very near each other (36). In contrast, these subdomains are distal to each other in p66.

We mapped the RT-binding region on IN to the carboxy-

terminal domain between residues 201 and 288. These results have been corroborated by the studies of Zhu et al. (39), which showed that the carboxy-terminal domain is sufficient to interact with RT and that a C130S mutation blocks this interaction. The C-terminal domain has a nonspecific DNA-binding activity and is also known to be involved in multimeric interactions (1, 3, 13, 20, 25). It is known that multimerization of IN is necessary for its integration activity (9, 10, 12, 18, 35). We have found that a mutant of IN (V260E) defective for IN-IN interactions (20) is not defective for RT-IN interactions. These results suggest that (i) the oligomerization of IN may not be necessary for RT-IN interaction and (ii) the surface of the C-terminal domain necessary for IN-IN interactions may be distinct from the region necessary for RT-IN interactions.

Studies of HIV RT and IN demonstrate that they behave differently in vitro and in vivo. For example, in vivo, RT can efficiently synthesize ~10-kb DNA, whereas in vitro it can only synthesize a few hundred nucleotides. Similarly, in vitro reactions of IN are less efficient and require a high input of purified IN protein. These differences between in vivo and in vitro activities are most likely due to the milieu of the nucleoprotein complexes within the virions and possibly to the presence of other viral and/or cellular proteins. Thus, it is likely that these two Pol-derived interacting proteins have an influence on each other's activities. This conclusion is supported by earlier results showing that several mutations in HIV-1 IN protein blocked viral replication at the level of reverse transcription (38) and by the recent observations that the C130S mutation in IN results in a block to reverse transcription (39).

Our results with testing the effect of IN on RT activity demonstrated that the RNA-dependent DNA polymerase (RDDP) activity of HIV-1 RT, or its processivity, was unaffected by the presence of the IN protein. Other observations indicate inhibition of RT activity by the IN protein. For example, Tasara et al. showed that the presence of IN inhibits DNA-dependent DNA polymerization by HIV-1 RT but not RDDP activity (32). The latter results are in agreement with our results showing that IN protein does not influence RNA-dependent DNA polymerization by RT. Another report by Oz et al. also indicated that IN had no influence on RT activity or processivity (26). The differential effect on RNA- versus DNA-dependent DNA polymerization activities may be due to the ability of IN to bind DNA but not RNA, resulting in competition for the same substrate by both the RT and IN proteins. Thus, it is formally possible that the influence of IN mutations on reverse transcription in infected cells, as implied by Wu et al. (38) and Zhu et al. (39), is indirect, involving the recruitment of other viral or host proteins (see below).

Our results indicate that RT has no effect on the 3′ processing activity of IN at lower concentrations but that at higher concentrations it inhibits this activity. This is most likely the result of RT competing with IN for the oligonucleotide substrates used for binding. The molar ratios used ranged from 1:0.03125 to 1:2 (IN monomer to RT heterodimer). If we assume IN to be a tetramer, then the ratio would be 4:1 to 1:16 (IN tetramer to RT heterodimer). Thus, 3′ processing is completely inhibited by RT when the RT-to-IN ratio exceeds 1 to 1 (Fig. 9B, lanes 9 to 12). The inhibition of 3′ processing by IN in the presence of RT is in agreement with the results of Tasara et al. (32) and Oz et al. (26).
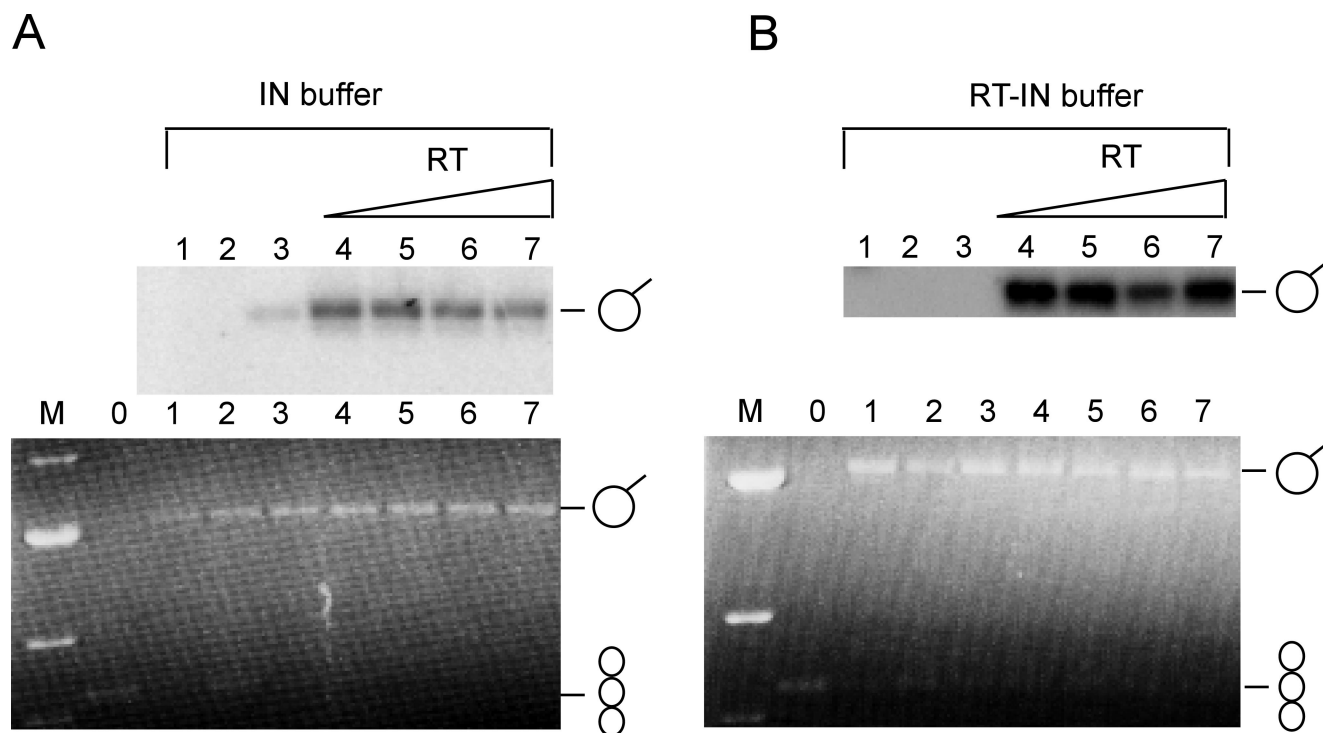
FIG. 10. Effect of RT on the joining reaction by IN. (A) Effect of RT on joining reactions by IN using standard IN buffer. (Top) Autoradiogram displaying products of joining reactions carried out in the presence of increasing concentrations of RT. Lane 1, no IN; lane 2, RT alone (4.68 pmol); lane 3, IN alone (4.68 pmol); lanes 4 to 7, 4.68 pmol of IN with various proportions of RT at molar ratios (IN monomer to RT heterodimer) of 1:0.125, 1:0.25, 1:0.5, and 1:1, respectively. (Bottom) Agarose gel of the same joining reactions. Lane M, markers showing 4-, 3-, 2-, and 1.5-kb bands; lane 0, 0.2 μg of uncut pBluescript. Lanes 1 to 7 are identical to lanes 1 to 7 above. The starting supercoiled DNA substrate (bottom only; indicated with a twisted circle symbol) and the nonconcerted joining reaction products (top and bottom; indicated with a panhandle symbol) are shown. (B) (Top and bottom) Exactly as described for panel A, except that the reactions were carried out in the presence of RT-IN buffer and the marker lane (M) shows only 3-, 2-, and 1.5-kb bands.

Perhaps the most dramatic effect we observed was the stimulation of the strand transfer activity of IN by RT. Under standard IN assay conditions, RT stimulated joining 5-fold, but in buffer optimized for RT-IN interaction, the effect was dramatic (up to 155-fold). The large stimulation of joining by RT observed here was not previously reported. This was most likely the result of differences in the assays used and the use of buffer conditions optimized for RT-IN interaction. Both Tasara et al. (32) and Oz et al. (26) used radiolabeled oligonucleotide substrates to measure integration as a measure of joining activity and observed inhibition by RT. We, too, used radiolabeled IN oligonucleotide substrates, but we measured their integration into supercoiled plasmid DNA. Using the same assay, Carteau et al. also observed stimulation of joining activity by RT (although at a lower level) (6). These authors reported that the addition of p51 stimulated joining by IN, which is in agreement with our finding that p51 preparations bind to the IN protein as efficiently as p66 and p66/p51 heterodimers. We believe that the presence of a bivalent IN-binding protein, such as RT, in the reaction mixture may promote multimerization of IN, increasing the effective concentration of the active form, thus leading to stimulation of joining by IN. A similar effect on IN activity had been observed before, when another IN-binding protein, INI1, was present in the reaction mixture (19). At lower concentrations of IN, INI1 stimulated the activity, and at higher concentrations, it had no

effect or had inhibitory activity (19). Thus, the effect of IN-interacting proteins on its activity again suggests that high efficiency of IN activity in vivo can be attributed to the milieu of IN-interacting proteins, including RT, binding to it and modulating its activity.

Wu et al. reported a significant block to viral reverse transcription caused by the mutations at conserved residues of IN, suggesting that IN interactions with other viral proteins, such as RT, may be important for reverse transcription in addition to integration. This belief is in agreement with the report that reverse transcription in vivo is more efficient than in vitro (17). It is possible that IN is required for recruiting cellular proteins that may be essential to initiate reverse transcription in the intracellular reverse-transcription complexes. It is important to reconcile two important differences between the biochemical findings in this report and the virological findings of Wu et al., who studied the functional effects of RT-IN interaction (38). First, in our GST pull-down assays, the presence of the mutations H12A, H16A, D116A, and F185A in the IN protein did not affect the association between IN and RT. Furthermore, the RT-binding domain of IN resides in the carboxy-terminal domain, which was also corroborated by Zhu et al. (39). It is possible that mutations in the N-terminal Zn-binding domain of IN influence the RT-IN interaction in vivo within the architecture of PICs but are unable to affect the interaction in solution. It is also possible that mutations in the Zn-binding

domain affect the association of IN with another viral or cellular protein, which may mediate the IN effect on viral reverse transcription. Second, in spite of the block to reverse transcription caused by IN mutations during viral replication, the in vitro association of IN with RT did not lead to any stimulation of RDDP activity in our hands. In fact, two groups have shown inhibition of DNA-dependent DNA polymerase activity of RT by IN. It is possible that this inhibition merely represents competition for nonspecific binding to the same DNA by two DNA-binding proteins. This inhibition may not be observed in vivo, since the viral DNA is likely coated by the nucleocapsid protein, minimizing nonspecific binding.

In order to highlight the significance of the interaction for virus replication, it would be necessary to study mutants that disrupt RT-IN interaction without affecting their enzymatic activities. Unfortunately, the two potential RT interaction-negative IN mutants (W235A and W235E) reported by Ishikawa et al. (15) displayed wild-type levels of interaction with RT in our repeated attempts using two different fusion partners. The C130S mutation, which abolished interaction with RT and led to virions defective for reverse transcription (39), offers a useful avenue for further studies. These results are reminiscent of the reports that several mutations in the HIV-1 IN protein did not affect in vitro IN or RT activity but displayed an in vivo DNA synthesis defect (22). Our results and those of Zhu et al. together lay a foundation for further studies aimed at understanding the dynamics of RT-IN interactions during viral reverse transcription in vivo.

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